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SHORT COMMUNICATION

Differential expression of the integrins α6Aβ4 and α6Bβ4 along the crypt–villus axis in the human small intestine

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Abstract The integrin α6 subunit exists as two different variants, termed α6A and α6B. These two variants have been shown to harbor potentially distinct biochemical properties but little is known about their cellular function. The aim of this work was to characterize the expression of the integrin α6A and B variants in relation to cell proliferation and differentiation in the human small intestinal epithelium. The results showed distinct expression patterns for the two variants along the crypt–villus axis. Indeed, proliferative cells of the crypt were found to predominantly express α6A, while differentiated enterocytes and Paneth cells expressed the α6B variant. A similar relationship was observed in intestinal cell models by competitive RT-PCR. Further studies in the Caco-2 cell model showed that manipulating the cellular balance of the two α6 variants can influence transcriptional activities related to cell proliferation but not differentiation. This suggests that differential expression of the α6 subunits is involved in the intestinal epithelial cell renewal process. Further studies will be needed to substantiate this hypothesis.

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Abbreviations

ECM	Extracellular matrix
BM	Basement membrane
RLU	Relative luciferase units

Introduction

Integrins are a family of heterodimeric transmembrane receptors that, besides providing a physical link between the basement membrane (BM) and the cytoskeleton of epithelial cells, act as platforms for intracellular signaling as a consequence of ligand binding and cross talk with receptor tyrosine-kinases (RTKs) (Giancotti and Tarone 2003). To date, 18 α and 8 β subunits have been identified in the human, leading to the formation of at least 24 distinct functional receptors. However, extensive alternative splicing and post-translational modification of both groups of subunits leads to the generation of considerably more forms in vivo (de Melker and Sonnenberg 1999). The α6 subunit mRNA undergoes alternative splicing yielding two distinct isoforms (Hogervorst et al. 1991), termed α6A and α6B, with distinct cytoplasmic domains and dissimilar patterns of expression throughout the human organism (Hogervorst et al. 1993). The A variant has been reported to be the only variant expressed in the mammary gland, peripheral nerves and basal keratinocytes while the B variant is predominant in the kidney. The intestine was initially reported to express both variants (Hogervorst et al. 1993). These patterns of expression for α6A and α6B as well as their dissimilar temporal expression during

embryonic development (Thorsteinsdottir et al. 1995) may imply that they serve different biological functions.

In the human intestine, the α6 subunit dimerizes with the β4 subunit forming the α6β4 integrin (Basora et al. 1999). The relatively simple structural and functional renewal unit of the small intestine, the crypt–villus axis, makes it an attractive model for the study of epithelial cell proliferation and maturation (Babyatsky and Podolsky 1995). Positional control of the enterocytes and their subsequent function is controlled by cell–cell and cell–extracellular matrix (ECM) interactions with the underlying BM (Teller and Beaulieu 2001). The importance of the latter is exemplified by the inductive effects on enterocytic cytology by specific laminin variants (Vachon and Beaulieu 1995; Virtanen et al. 2000), while analysis of several molecules involved in cell–ECM interactions, including integrins, has revealed distinct patterns of expression along the crypt–villus axis in relation to the differentiation state of enterocytes (Beaulieu 1997; Teller and Beaulieu 2001). Furthermore, the α6β4 integrin has been shown to be an important player in mediating migration and invasion of colon cancer cells (Lohi et al. 2000; Mercurio and Rabinovitz 2001; Ni et al. 2005; Pouliot et al. 2001).

In the present work, in order to further characterize the role of the integrin α6 subunit in human epithelial cell biology, we have investigated the expression patterns of the two splice variants along the crypt–villus axis of the small intestine and in intestinal cell models. We have found that the α6A variant is predominantly expressed by undifferentiated cells where it may play a role in the modulation of proliferation while the α6B variant is mainly detected in differentiated intestinal cells.

Materials and methods

Tissues

Primary tissues of healthy adult ileum were obtained from Quebec Transplant (Quebec, Canada). Primary extracts of fully differentiated villus enterocytes were obtained according to a previously published protocol (Perreault and Beaulieu 1998). All tissues were obtained in accordance with protocols approved by the local Institutional Human Research Review Committee. The preparation and embedding of tissues for cryosectioning and RNA extraction was performed as described previously (Ni et al. 2005).

Primary antibodies

An antibody recognizing both splice variants of integrin α6 (G0H3) (Sonnenberg et al. 1987) and antibodies recognizing α6A (1A10) and α6B (6B4) (Hogervorst et al. 1993) were originally generous gifts from Dr. A. Sonnenberg (Division of Cell Biology, The Netherlands Cancer Institute, Amsterdam). Subsequently, these antibodies were obtained from Santa Cruz (Santa Cruz, CA; G0H3), Chemicon (Temecula, CA; 1A10) and MUbio Products (Maastricht, The Netherlands; 6B4). A rabbit polyclonal α6A (α6-cytoA) (de Curtis and Reichardt 1993), a kind gift from Dr. de Curtis (Department of Molecular Pathology and Medicine, San Raffaele Scientific Institute, Milan, Italy), anti-Ki67 (KiS5, Chemicon), anti-lysozyme (DAKOCytomation) and anti β-actin (C4, Chemicon) were also used.

Indirect immunofluorescence

Cryosections were fixed in 2% paraformaldehyde for the detection of α6, α6A, Ki67 and lysozyme or in −20°C ethanol for the detection of α6B and processed as described previously (Ni et al. 2005). In all cases, no immunofluorescent staining was observed when a mix of mouse and rabbit non-immune sera replaced primary antibodies.

Western blot

Western blots were performed as SDS-PAGE under non-denaturing conditions as previously described (Ni et al. 2005). After transfer of the separated samples to a nitrocellulose membrane (BioRad, Hercules, CA) unspecific protein binding to the membrane was blocked by 2% BSA/0.1% Tween followed by incubation with the α6A 1A10 monoclonal antibody. Following detection, the membrane was stripped of antibody by incubation in stripping solution [50 mM Tris (pH 6.8), 2% SDS, 100 mM β-mercaptoethanol] at 50°C for 20 min after which the membrane was reprobed with the α6B 6B4 antibody using 2% BSA/0.1% Tween as blocking solution. Finally, the membrane was restripped and reprobed with a β-actin antibody in 5% skim milk powder/0.1 % Tween as an input control.

Plasmids and plasmid construction

The β-catenin/TCF4 responsive luciferase reporter plasmid, TOPFlash (Upstate, Charlottesville, VA) has been characterized elsewhere (Korinek et al. 1997). Firefly luciferase reporter plasmids carrying promoters of the differentiation markers lactase-phlorizin hydrolase (pGL3-LPH1085-13910T) (Troelsen et al. 2003), intestinal alkaline phosphatase (pALPI_566) (Olsen et al. 2005) and sucrase-isomaltase (pSI-202/+54) (Boudreau et al. 2002) have been characterized elsewhere. The dipeptidyl peptidase IV (DPPIV) promoter plasmid was generated in our lab by PCR-amplification of 1,382 bp of the immediate 5′ promoter of DPPIV (sense primer: 5′-CGGGGTACCTTGGAAGAGGGAGGAGGAG-3′, antisense primer: 5′-GAAGATCT

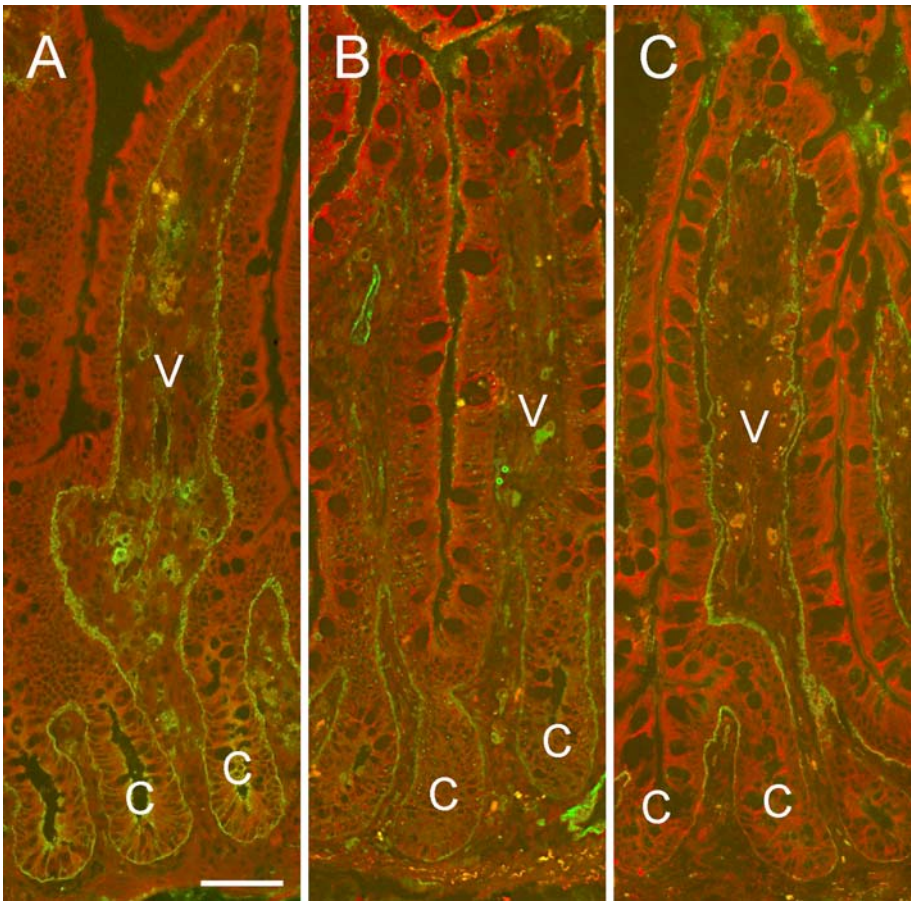

Fig. 1 Representative immunofluorescent staining on tissue sections of the adult small intestinal mucosa for detection of the α6 integrin subunit and its α6A and α6B splice variants. **a** A common α6 epitope was detected at the base of both crypt (*c*) and villus (*v*) cells. **b** The A variant was predominantly located in the crypts (*c*). **c** The B variant was detected in the villus (*v*) and upper and lower thirds of the crypt (*c*). Red-brown signal: Evan blue counter stain. Magnifications **a**–**c**: *scale bar* in **a** = 50 μm

AGTCACTCGCCGCTGGCA-3′) followed by *Kpn*I and *Bgl*II (underlined sequences) mediated insertion into pGL3, yielding the plasmid pGL3/Prom.dppIV. An expression vector containing the cDNA of integrin α6A, pRc/CMV-α6A (Delwel et al. 1993), was a generous gift from Dr. Sonnenberg (Division of Cell Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands). For α6B, the cDNA encoding the cytoplasmic tail of the integrin α6A subunit in pRc/CMV-α6A was replaced by the cDNA encoding the cytoplasmic tail of the integrin α6B subunit by *Xba*I digestion of the recipient (pRc/CMV-α6A) and donor (pPCR-Script-α6B) vectors followed by ligation, generating pRc/CMV-α6B.

Cell culture

The crypt-like human intestinal epithelial HIEC cells and Caco-2/15 cells were grown as described previously (Basora et al. 1999; Perreault and Beaulieu 1996; Vachon and Beaulieu 1995).

RT-PCR

Primers used to co-amplify the α6A and α6B transcripts were sense: 5′-CTAACGGAGTCTCACAACTC-3′ and antisense: 5′-AGTTAAAACTGTAGGTTCG-3′. Each cycle was composed of template denaturation at 94°C for 1 min, primer annealing at 65°C for 1 min and elongation at 72°C for 1 min. The primer annealing temperature was decreased by 0.5°C after each round of amplification for 40 cycles followed by a final 15 cycles at an annealing temperature of 45°C.

Transfection and luciferase measurement

Caco-2/15 cells were transfected using FuGENE transfection agent (Roche, Indianapolis, IN). Firefly and renilla luciferase activities were measured using the Dual-Luciferase® Reporter Assay System (Promega Corporation, Madison, WI) according to the manufacturer's instructions as described previously (Escaffit et al. 2005).

Results and discussion

As shown previously for the β4 subunit (Basora et al. 1999), immunodetection of the α6 integrin subunit using an antibody directed against the extracellular domain (G0H3) (Sonnenberg et al. 1987) yielded ubiquitous staining at the base of the epithelial cells in both villus and crypt (Fig. 1a). Staining for the integrin α6A subunit was observed in the

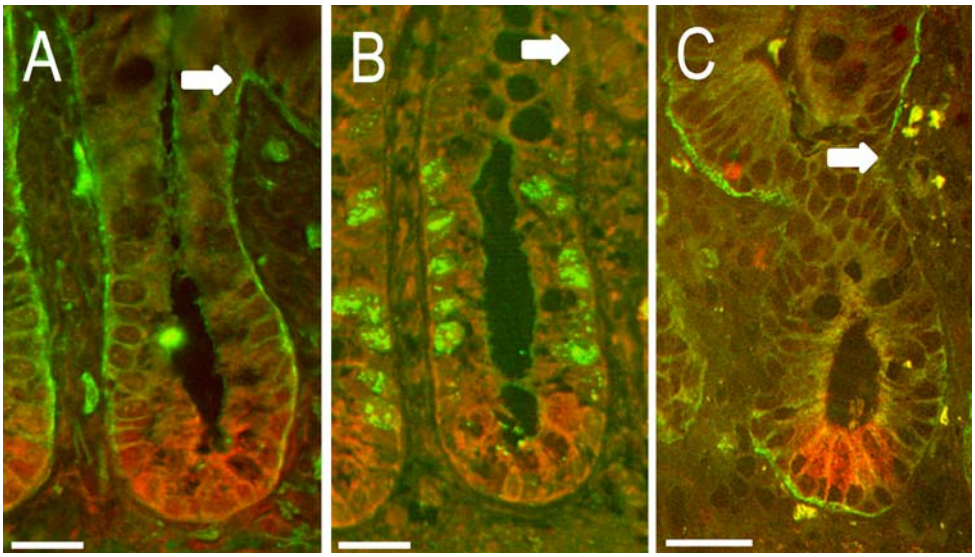

Fig. 2 Representative double immunofluorescent staining on tissue sections of adult small intestinal crypts for detection of α6A (**a**), Ki67 (**b**), α6B (**c**) in *green* and lysozyme in *red* (**a**–**c**). Predominant distribution of the α6A subunit was found in the middle part of the crypt (**a**), above the Paneth cell region as determined with lysozyme immunostaining and adjacent to the Ki67-positive region as determined in the corresponding crypt from a serial cryosection (**b**). The α6B subunit was found to be predominant in the upper crypt/lower villus region as well as in the bottom of the glands (**c**), a region that contains Paneth cells as identified with lysozyme. *Scale bars* 25 μm

epithelium and was found to be restricted to proliferative cells in the lower–middle to upper crypts with a fade out of staining at the base of the villus (Fig. 1b, Fig. S1A). Additional labeling was seen in the vasculature of the lamina propria. In contrast, staining for the α6B variant was found to be predominant at the base of villus epithelial cells and at the bottom of the crypts, while relatively weak staining was detected in the middle to upper regions of the crypts (Fig. 1c, Fig. S1B). The relation of α6A and α6B expression to intestinal cell proliferation and differentiation, respectively, was confirmed by double staining with specific proliferation and Paneth cell markers. As shown in Fig. 2, expression of the α6A subunit was found to be above the Paneth cell region as determined with lysozyme co-immunostaining (Fig. 2a) and adjacent to the rich Ki67-positive region as determined in the corresponding crypt from serial cryosections (Fig. 2b). In contrast, expression of the α6B subunit was predominantly detected in the upper crypt/lower villus region and co-localized at the bottom of the crypts with differentiated Paneth cells as identified by lysozyme (Fig. 2c). This pattern of expression was consistently observed and the images shown are representative of the six samples studied.

We then performed competitive RT-PCR using primers that amplify the transcripts of both α6 variants from cDNA originating from the normal crypt-like human cell line HIEC and primary human villus epithelial cells, as well as from the Caco-2/15 cell line that undergoes an intestinal differentiation program at postconfluence. A clear shift from a high α6A/α6B transcript ratio to a low ratio was seen accompanying differentiation at different stages of enterocytic differentiation [Fig. 3a; statistically significantly different from SC, $P < 0.05$, Tukey's One Way Analysis of Variance (ANOVA), $n = 3$]. A similar shift was observed in Caco-2/15 cells at the protein level [Fig. 3b; statistically significantly different from SC, $P < 0.01$, Tukey's One Way Analysis of Variance (ANOVA), $n = 3$]. The findings that the two splice variants of the α6 integrin are differentially expressed in the proliferative and differentiated compartments of the gut, and that there is an association between the ratio of these two splice variants with the stage of differentiation, are consistent with previous findings of distinct expression of components of the integrin-ECM system in gastrointestinal biology (Basora et al. 1997, 1999) supporting the importance of interactions between the epithelium of the intestinal tract and the underlying BM (Beaulieu 1997; Teller and Beaulieu 2001).

Distinct patterns of expression for the two α6 variants have been reported in different organs during development (de Curtis and Reichardt 1993; Segat et al. 2002; Thorsteinsdottir et al. 1995) suggesting a functional importance of the expression ratio of the two forms. The exclusive expression of α6A in a rapidly dividing cellular compartment is known from the epidermis (Hogervorst et al. 1993). Interestingly, it has been suggested that the ratio of the two variants can determine cellular behavior and that a proper cellular response is dependent on the presence of both variants rather than a substitution of one with the other (Segat et al. 2002). In agreement with this, we observed a modulation of the α6A/α6B ratio in intestinal cells rather than the replacement of one α6 variant with the other.

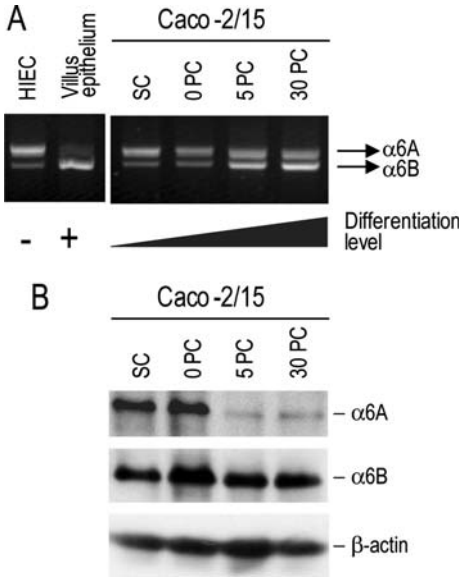

Fig. 3 Competitive RT-PCR of splice variant expression and western blot analysis showing down-regulation of the α6A variant upon cell-cycle exit in intestinal cells. **a** Representative competitive RT-PCR results. **b** Representative western blot analysis. HIEC: proliferative crypt cells; Villus epithelium: extracts of differentiated human villus cells; Caco-2/15: proliferative at sub-confluence (*SC*) while differentiating at post-confluence (*PC*)

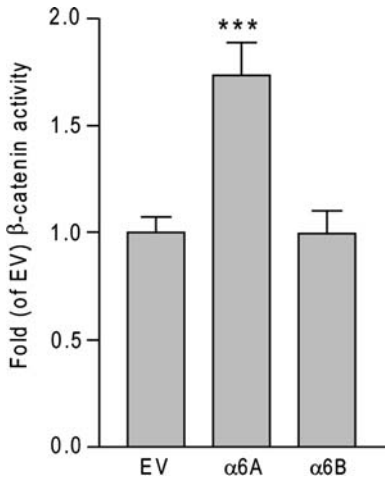

Fig. 4 Response of promoter activities associated with proliferation. Representative experiment of β-catenin/TCF promoter activity in response to co-transfection with either α6A or α6B expression vectors in 40–60% confluent Caco-2/15 cells. Empty vector (EV) was used as control. Mean ± SEM. ***: statistically significantly different from EV, $P < 0.001$, Tukey's One Way Analysis of Variance (ANOVA)

An immediate question arising from the distinct expression patterns in the different epithelial compartments of the intestine is whether the high α6A/α6B ratio in the proliferative zone is of functional importance for proliferation or whether a low α6A/α6B ratio in the differentiated zone is permissive for enterocytic differentiation. First, to verify whether the reduction of the α6A/α6B ratio was related to differentiation, Caco-2/15 cells were co-transfected with reporter vectors carrying promoters of the enterocytic differentiation markers sucrase-isomaltase, intestinal alkaline phosphatase, lactase-phlorizin hydrolase or dipeptidyl peptidase IV (DPPIV) and expression vectors encoding α6A (pRc/CMV-α6A) or α6B (pRc/CMV-α6B). As illustrated with DPPIV (Fig. S2), independent experiments revealed no differential activation of any of the four tested promoters in newly confluent Caco-2/15 cells. These results suggest that α6Bβ4 is not involved in the initiation of differentiation although a role in modulating differentiation at later stages or regulating other functions such as cell migration cannot be excluded. Conversely, modulation of the early steps of intestinal cell differentiation observed in response to cell–laminin interactions (Vachon and Beaulieu 1995) could be mediated by other laminin receptors such as the α7Bβ1 integrin (Basora et al. 1997).

Second, we tested the ability of the two variants to differentially affect intracellular pathways associated with enterocytic proliferation by performing co-transfections of the two integrin α6 variants with a reporter plasmid responding to β-catenin/TCF (TOPFlash) activity. This activity is associated with cell-cycle progression (Korinek et al. 1997). The β-catenin/TCF complex was found to be significantly and specifically stimulated by α6A (Fig. 4) suggesting a link between α6A expression and promotion of cell proliferation. However, stable expression of the α6A subunit in Caco-2/15 cells did not result in a net increase in cell proliferation (Dydensborg et al. unpublished data) suggesting that the β-catenin pathway linked to the regulation of cell proliferation in this APC-mutated colon cancer cell line (Ilyas et al. 1997) is already at maximal stimulation.

We have previously demonstrated that the α6β4 integrin is the only α6 containing integrin in the human intestine (Basora et al. 1999). In this context, it is noteworthy that there is substantial evidence for the differential capacity of the α6Aβ1 and α6Bβ1 integrins to initiate intracellular signaling (Shaw et al. 1995; Wei et al. 1998) and facilitate migration on laminin (Shaw and Mercurio 1995), but to our knowledge, no study has ever demonstrated a functional difference between the α6Aβ4 and α6Bβ4 integrins, making the present work the first to demonstrate such a difference. The finding that the α6Aβ4 integrin is predominant in intestinal proliferative cells both in the intact intestine and in established intestinal cell lines suggests that the α6A/α6B ratio plays an important role in intestinal homeostasis. This interesting possibility should be directly investigated in the future.

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